ANDROLOGY
Original Article

EBV induced loss of sperm quality

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ABSTRACT

Objective: To analyze the presence of Epstein–Barr-virus (EBV) in sperm samples from patients diagnosed with some impairment of the fertility parameters evaluated using seminogram and to observe if there is any difference with the normozoospermic samples. We hypothesize that an EBV infection is responsible for the upregulation of the miRNA 199-3p, which binds to the 3'UTR of endothelin-1 (ET-1). ET-1 is a key factor to produce Vimentin (Vim3), and therefore, it influences the expression of Vim3. Since Vim3 is predominantly detectable in sperms without any structural defects, the newly identified regulation mechanism can be responsible for the loss of sperm quality.

Material and methods: This study was performed from January 2017 to December 2020 and included 27 patients who provided ejaculated samples obtained by masturbation. Ejaculates were evaluated according to the Word Health Organization's criteria. Posteriorly, the samples were sorted according to the seminogram diagnosis and further analyzed using different enzyme-linked absorbed immune assays to determine the level or concentration of Epstein–Barr nuclear antigen (EBNA), ET-1, and Vim3.

Results: All sperm samples with the impairment of fertility parameters contained the EBNA and presented a downregulation of ET-1 and Vim3. In addition, sperms located in the swim ups are also partially positive for the EBV virus in different clinical aspects.

Conclusion: Based on the regulation mechanism here presented, it seems that the EBV induces changes at the miRNA level, which are responsible for the decreasing of sperm quality.

Keywords: Endothelin-1; herpesvirus 4; human infertility; male; vimentin.

Introduction

Infertility is a major condition in men worldwide, approximately 70 million men suffering from this condition.[1,2] Overall, 8-12% of couples are affected by this condition, of which 50% are due to the "male factor." These "male factors" are associated with the concentration of the sperms and alteration in the sperm morphology and motility.[2] There are many different causes of infertility; however, the most common causes are gonad disorders (30-40%), disorders affecting sperm transport (10-20%), and hypothalamus and pituitary gland disorders (1-2%). Nevertheless, 50-60% are due to unknown causes.[1,3] Furthermore, sperm disorders can also be caused by different factors, like inflammation of the testis, abnormally developed testis, genetic disorders, or hormone problems.[4]

Epstein–Barr virus (EBV) is a double-stranded DNA virus. This virus is primarily known to cause infectious mononucleosis with a prevalence of 90% belongs to one of the most common infections worldwide.[5,6] In addition, EBV and other herpes viruses can be associated with infertility. It was recently shown that EBV is present in seminal fluid.[7]

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Endothelin-1 (ET-1) is a multifunctional protein and involved in many different signal transduction pathways. It is known that ET-1 induces the production of truncated proteins via a transcriptional stop.[8] These truncated proteins are shorter than their full-length variants and biological active. Maggi et al. already described that ET-1 and the two corresponding receptors, called ETAR and ETBR, are present in human testis cells, especially in Sertoli cells.[9] They already hypothesized that the ET-1 expression in seminal fluid can be probably used as Sertoli function marker.[9] Besides being present in the testis, it is also known that ET-1 and its corresponding receptors are also found in the seminal fluid.[10]

The truncated variant of Vimentin, called Vim3, is a marker for the differentiation of normozoospermia and oligoasthenoteratozoospermia (OAT) syndrome.[3] Full-length Vim3 is a structural protein, which is predominantly found in the head domain of sperms with a normal mobility and appearance. In contrast to that Vim3 is mainly found in the middle part of sperms.[3]

We hypothesize that an EBV infection is responsible for the upregulation of the miRNA 199-3p, which binds to the 3'UTR of ET-1. ET-1 is a key factor to produce Vim3, and therefore, it influences the expression of Vim3.[8]

Since Vim3 is predominantly detectable in sperms without any structural defects, the newly identified regulation mechanism can be responsible for the loss of sperm quality.

Material and Methods

Patient Information and Samples

The patient's ejaculated (n = 27) samples were analyzed and categorized according to the nomenclature of the World Health Organization (WHO) from 2010. After categorizing the ejaculated samples, those were immediately analyzed. This study complies with the Declaration of Helsinki, and a local ethics committee approval was obtained from the BioMASOTA, University Hospital of Cologne (file reference 12-163). All patients signed the informed consent (BioMASOTA, file reference 12-163). Samples were collected from January 2017 to December 2020. All patients were seeking for fertility medical advice due to the primary infertility at the andrology department; in all of them, a secondary cause was excluded, and in the case of the normozoospermia group, the collection was from couples with an infertility female factor diagnosis. The median age at the time of samples provided was 32.9 ± 2.1 years old—none of them reported having any chronic medical conditions and none of them were under treatment for any purpose. Cryopreservation was performed following the slow freezing technique[11] and thawed took place at room temperature (RT) for 15 minutes. The patient samples are listed in Table 1.

Main Points

- Epstein–Barr virus has an impact on the regulation of Vim3.
- Vim3 production is induced by the expression of ET-1.
- Vim3 is a marker for the differentiation between normozoopermia and OAT syndrome.
- EBV seems to have an impact on sperm quality.

Enzyme-Linked Absorbed Immune Assay

Epstein–Barr nuclear antigen (EBNA), ET-1, and Vim3 levels were determined using enzyme-linked absorbed immune assay (ELISA). The procedure performed was previously described in Funke et al.[3] The primary antibodies in EBNA (Santa Cruz Biotechnology Inc., Dallas, USA) and Vim3 (Davids Biotechnologie GmbH, Regensburg, Germany) in a dilution ratio of 1:1,000 were used and incubated for 1 h at RT. The secondary antibody antimouse-conjugated HRP (Biomol GmbH, Hamburg, Germany) was used in a 1:5,000 dilution and incubated for 1 h at RT. For the determination of the ET-1 expression, an ET-1 ELISA kit (Wuhan Fine Biotech Co. Ltd., Wuhan, China) was used according to the manufacturer's protocols.

RNA Isolation

For the RNA isolation, the sperms were washed three times with 1×PBS to remove the seminal fluid. The isolation was performed using the miRNeasy kit (Qiagen) according to the manufacturer's protocols. Quantification of the isolated RNA was done using the NanoDrop technology.[12] The isolated total RNA samples were stored at –80°C for further use.

Reverse Transcriptase Polymerase Chain Reaction

Reverse transcriptation was obtained from 500 ng total RNA using miRScript kit according to the protocol (Invitrogen, Carlsbad, USA).

Quantitative Polymerase Chain Reaction (qRT-PCR)

One microliter of the reverse transcript product was used for the real-time PCR analysis. For quantitative analysis, β-actin or 5s rRNA was measured. The procedure was previously described by von Brandenstein et al.[13]

Statistics

For the statistical analysis, the Prism 5 (GraphPad Software, San Diego, USA) software was used. T-test was performed, and significant differences were calculated ($^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$). All experiments were performed triplicate.

Table 1. Overview of the Patient Data

Seminogram diagmosis	N	Age (years) (mean ± SD)	Sperm concentration (millions mL^{-1}) (mean ± SD)	Volume (mL) (mean ± SD)	Total concentration (millions) (mean ± SD)	Mobility a + b (%) (mean ± SD)	Mobility c (%) (mean ± SD)	Mobility d (%) (mean ± SD)	%Normal morphology (mean ± SD)
Normozoospermia	5	28.6 ± 3.9	227 ± 116.48	2.9 ± 0.571	719.4 ± 466.545	61.4 ± 7.505	6.2 ± 3.739	32.8 ± 6.054	4.8 ± 0.392
OAT	5	30.6 ± 3.01	10.8 ± 2.86	3.24 ± 0.96	33.1 ± 7.75	23.4 ± 5.13	4.8 ± 3.58	70.2 ± 8.11	1.2 ± 0.96
Azoospermia	4	35.2 ± 3.18	0	2.38 ± 1.24	0	0	0	0	0
Teratozoospermia	5	33.2 ± 3.84	98 ± 44.98	3.2 ± 1.52	330.6 ± 207.39	56.25 ± 6.11	2 ± 1.49	41.75 ± 6.37	2 ± 0.87
Asthenoteratozoospermia	4	38.5 ± 8.41	53.5 ± 33.20	2.475 ± 0.73	142.8 ± 114.86	19 ± 5.88	5.3125 ± 2.75	75.6875 ± 3.20	1.875 ± 1.01
Oligozoospermia	4	32.25 ± 1.674	3.55 ± 2.75	1.875 ± 0.64	4.5067 ± 3.94	43 ± 0	0.5 ± 0.98	56.5 ± 0.98	5 ± 1.96

N, number of samples; OAT, oligoasthenoteratozoospermia; SD, standard deviation.

Results

For the identification of the induced loss in sperm quality by EBV, the expression of the EBNA was measured in semen samples categorized according to the WHO. All sperm abnormalities presented a significant increase in EBNA in comparison to normozoospermia as well as teratozoospermia (Figure 1).

Since EBNA is responsible for the overexpression of miRNA 199-3p, this miRNA was analyzed in all samples. An overexpression of miRNA 199-3p was found in patient samples suffering from the OAT-syndrome. No significant increase could be detected in patients with teratozoospermia and asthenospermia (Figure 2).

Since we know that the miR-199-3p binds to the 3'UTR of ET-1 resulting in reduction, the ET-1 levels were determined in semen (Figure 3). An inverse level of expression between ET-1 and an existing EBV infection can be seen. The ET-1 expression was significantly downregulated in all samples compared to normozoospermia.

Based on the recently published regulation mechanism that ET-1 is a major key regulator in the production of Vim3, the

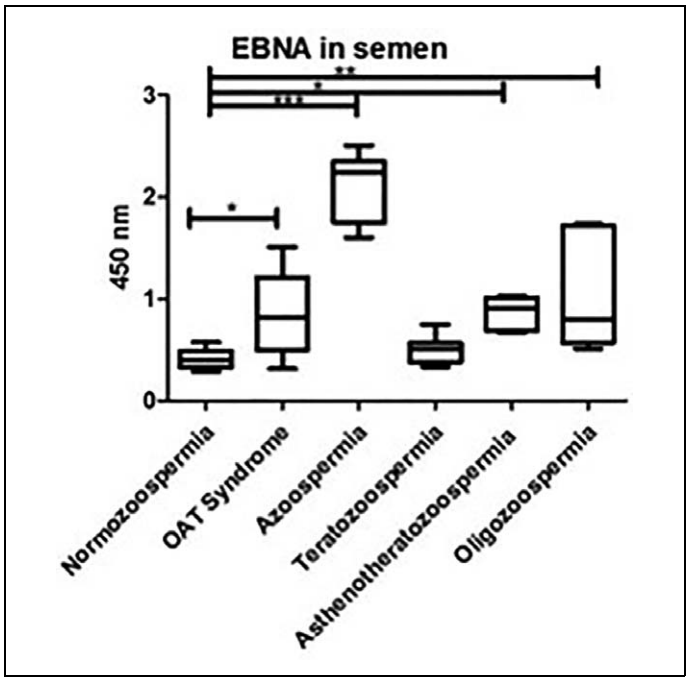

Figure 1. Epstein–Barr nuclear antigen (EBNA) expression in semen. All samples show an expression of EBNA. In the normozoospermia samples, the expression was significantly lower.

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expression of Vim3[8] was also determined via ELISA (Figure 4). It could be seen that the Vim3 expression in semen correlates with the ET-1 and EBNA expression. The highest amount of Vim3 was found in normozoospermia, indicating that Vim3 is useful for the differentiation of normozoospermia and the OAT syndrome.

Discussion

The relative high number of cases of unexplained male infertility[14] represents a big challenge as there is not possible to detect any alteration by routine semen analysis, which focuses mainly on spermatozoa count in ejaculation and their morphology and motility. The association between virus infection and male infertility has been previously investigated with variable non-conclusive results.[7,15] In healthy individuals, the EBV is highly prevalent, as it affects more than 90% of individuals worldwide.[6]

The association between EBV infections and its role in infertility is unknown. Based on the presented regulation mechanism, the presence of the EBNA antigen, which is only detectable after an EBV infection, might have an influence on the sperm quality. EBNA is the only viral protein that is produced in all phases of the infection.[16] It is known that an EBV infection is responsible for the overproduction of miR-199-3p,[17] which was also found in our sperm samples. All EBV positive samples show an overproduction of miR-199-3p. Furthermore, it is also known from the literature that this miR binds to the 3'UTR of ET-1 and is therefore responsible for the decreasing levels of this protein.[18] As recently published by our group, ET-1 is an important key regulator in the production of Vim3, since ET-1 upregulates the miR-498 which is responsible for the truncation of Vimentin to Vim3 on DNA level.[8]

Due to the decreasing levels of ET-1 in EBV-infected sperms, the Vim3 levels also decrease. Vim3 seems to be an important protein which is predominantly expressed in normozoospermia.[3] A downregulation of this protein is associated with decreasing the sperm quality. However, the exact role of Vim3 is still unknown. Nevertheless, Vim3 is a marker for the differentiation of normozoospermia and the OAT syndrome.[3]

The presented regulation mechanism can be an explanation for the unbalanced expression of Vim3 in normozoospermia and other clinical aspects. In ejaculates with less normal formed sperms, the differentiation between teratozoospermia and

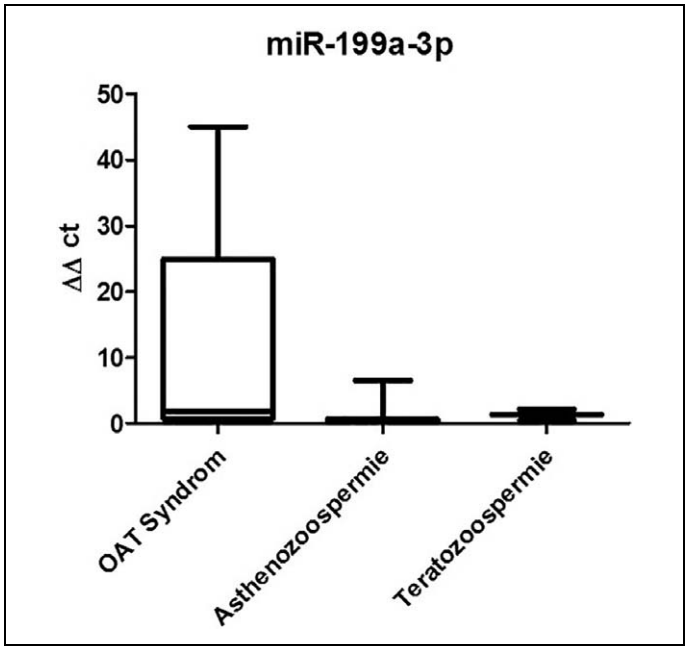


Figure 2. qRT-PCR results of the expression of the miRNA 199-3p. Patients with an OAT-syndrome present a significant increase in the expression of the miRNA 199-3p compared with normozoospermia patients (used as neutralizing standard). In samples from patients suffering under asthenozoospermie and teratozoospermia, a slight increase could be seen.

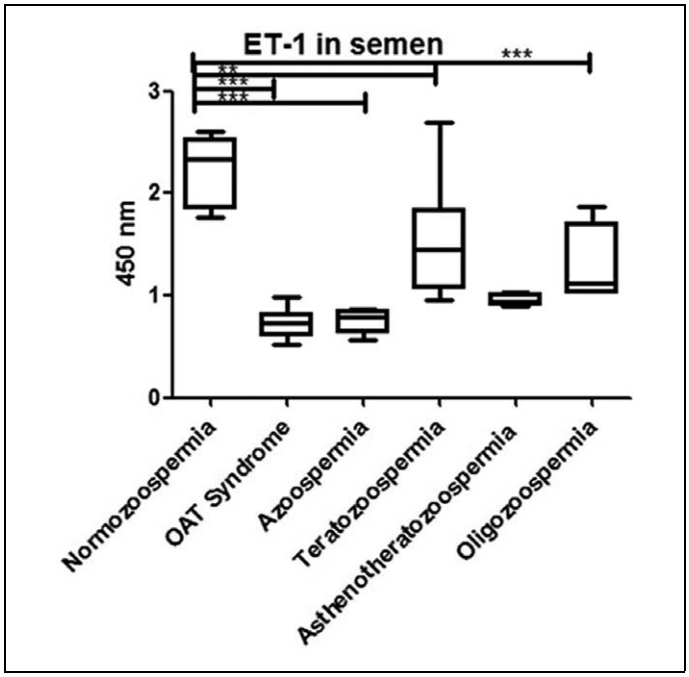
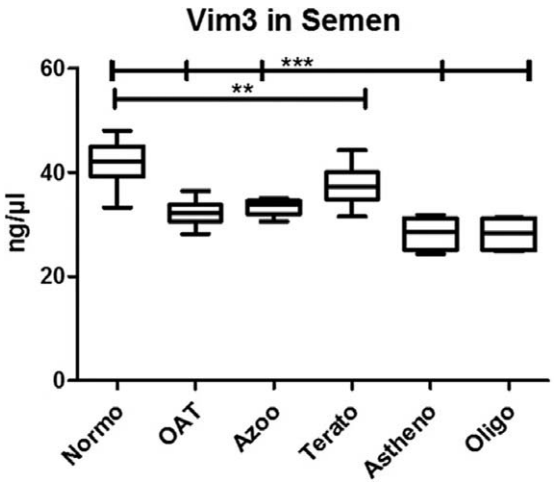


Figure 3. Endothelin 1 (ET-1) concentration in semen using ELISA. Here, the ET-1 concentration correlates with the concentration of EBNA in seminal fluid. A downregulation of ET-1 can be detected in all clinical situations, which present a significant upregulation of EBNA.



Normo vs OAT	***
Normo vs Azoo	***
Normo vs Terato	**
Normo vs Astheno	***
Normo vs Oligo	***
OAT vs Azoo	ns
OAT vs Terato	***
OAT vs Astheno	ns
OAT vs Oligo	ns
Azoo vs Terato	ns
Azoo vs Astheno	ns
Azoo vs Oligo	ns
Terato vs Astheno	***
Terato vs Oligo	***
Astheno vs Oligo	ns

Figure 4. Vim3 ELISA from semen. Vim3 concentration is significant lower in samples with fertility problems than in normozoospermia.

normozoospermia is very hard and frequently depends on the experience of the analyzer. Thus, it can occur that the results do not significantly differ from each other. Vim3 can be used as differentiation marker between normozoospermia and all other pathological ejaculates. It is necessary to corroborate in further studies the loss of fertilizing capacity of EBNA positive sperms since it is known that even patients with normozoospermia could have fertilizing potential issues. Thus, this could provide a reasonable explanation. It needs to be proven if the sperms from such patients are EBV positive. Concluding our results, it can be said that the presence of EBV in sperms has a significant influence on ejaculation and fertilization quality. The EBV-dependent downregulation of Vim3 is possibly responsible for a partial loss of quality.

Limitations

Although the results obtained show significant differences, further studies with a higher number of analyzed samples should be performed.

In future studies, sample sperms from normozoospermic patients with a proven paternity should be preferentially considered. Nevertheless, it may represent a challenge as most studies are being performed in samples obtained from fertility clinics. Therefore, in our normozoospermic group, we included only samples from couples with female factor infertility.

Ethics Committee Approval: Ethical committee approval was received from the BioMASOTA, University Hospital of Cologne (file reference 12-163).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Design - A.H., M.v.B.; Supervision - A.H., M.v.B.; Resources - M.H., J.H., P.P., J.D.; Materials - M.H., J.H., P.P., J.D.; Data Collection and/or Processing - M.H., J.T., B.K.; Analysis and/or Interpretation - M.H., M.v.B., B.K.; Literature Search - M.H., M.v.B., B.K., J.T.; Writing - M.H., J.T.; Critical Review - H.M., T.J., K.B., H.J., D.J., P.P., H.A., M.v.B.

Conflict of Interest: The authors have no conflicts of interest to declare.

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